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RESEARCH ARTICLE

N-WASP-directed actin polymerization activates Cas phosphorylation and lamellipodium spreading

Xian Zhang, Simon W. Moore, Thomas Iskratsch and Michael P. Sheetz*

ABSTRACT

Tyrosine phosphorylation of the substrate domain of Cas (CasSD) correlates with increased cell migration in healthy and diseased cells. Here, we address the mechanism leading to the phosphorylation of CasSD in the context of fibronectin-induced early spreading of fibroblasts. We have previously demonstrated that mechanical stretching of CasSD exposes phosphorylation sites for Src family kinases (SFKs). Surprisingly, phosphorylation of CasSD was independent of myosin contractile activity but dependent on actin polymerization. Furthermore, we found that CasSD phosphorylation in the early stages of cell spreading required: (1) integrin anchorage and integrin-mediated activation of SFKs, (2) association of Cas with focal adhesion kinase (FAK), and (3) N-WASP-driven actin-assembly activity. These findings, and analyses of the interactions of the Cas domains, indicate that the N-terminus of Cas associates with the FAK–N-WASP complex at the protrusive edge of the cell and that the C-terminus of Cas associates with the immobilized integrin–SFK cluster. Thus, extension of the leading edge mediated by actin polymerization could stretch Cas during early cell spreading, priming it for phosphorylation.

KEY WORDS: Crk-associated substrate, Cas, BCAR1, p130Cas, Src family kinase, SFK, Actin dynamics, Focal adhesion kinase, FAK

INTRODUCTION

The Crk-associated substrate (Cas, p130Cas or BCAR1) is a prominent member of the Cas family of multidomain scaffolding proteins that are phosphorylated by major tyrosine kinases of the Abl and Src family kinases (SFKs). The 15 YxxP motifs in the central substrate domain of Cas (CasSD) are the primary sites for phosphorylation by SFKs upon integrin-mediated adhesion or stimulation by various growth factors (Defilippi et al., 2006). Phosphorylation of CasSD plays an important role in cell migration, survival and transformation (Honda et al., 1999; Sanders and Basson, 2005; Shin et al., 2004; Wei et al., 2002; Auvinen et al., 1995). Phosphorylation of the YxxP motifs in CasSD creates docking sites for the SH2 domain protein Crk (Klemke et al., 1998; Vuori et al., 1996), which subsequently recruits guanine-exchange factors for the small GTPases Rac1 and Rap1 (Liu et al., 2007; Sharma and Mayer, 2008; Sawada et al., 2006; Tamada et al., 2004) that activate lamellipodium-based edge protrusion and adhesion formation (Boettner and Van

Aelst, 2009; Burridge and Wennerberg, 2004; Han et al., 2006). Thus, CasSD phosphorylation regulates cell motility, and the proposed consequences of the activation of Cas are consistent with the defects in motility observed in Cas-depleted cells (Honda et al., 1999; Sanders and Basson, 2005).

Cas is implicated in the rigidity response of the cell, and the level of phosphorylation of CasSD correlates with the rigidity of the matrix (Alexander et al., 2008; Kostic and Sheetz, 2006). Recent focus has been on Cas as the mechanosensor of matrix forces because stretch of the cytoskeleton, or an individual Cas molecule, exposes the CasSD, facilitates CasSD phosphorylation and triggers downstream biochemical reactions (Sawada et al., 2006; Tamada et al., 2004). For Cas to act as a mechanosensor, the substrate domain, which has 15 YxxP motifs, needs to be exposed by displacement of the N-terminus from the C-terminus of Cas, each of which is attached to mechanically separate portions of the cell. The multidomain Cas protein interacts with numerous proteins that link it to the cytoskeleton. The SH3 domain at the N-terminus of Cas binds to proline-rich sequences of focal adhesion kinase (FAK) and its autonomously expressed variant FRNK (Harte et al., 1996; Polte and Hanks, 1995). The C-terminus of Cas harbors binding sites for SFKs (Nakamoto et al., 1996). Binding of FAK and Src family kinases to the N- and C-terminus of Cas is important for the proper localization and phosphorylation of Cas (Fonseca et al., 2004; Hamamura et al., 2008; Nakamoto et al., 1997; Sanders and Basson, 2008). At the same time, FAK and SFKs are directly involved in rigidity sensing (Jiang et al., 2006; Kostic and Sheetz, 2006). An intriguing hypothesis that remains to be tested is whether FAK- and SFK-dependent phosphorylation of Cas requires the transduction of mechanical force to Cas through these binding partners.

In this study, we looked into the biochemical and mechanical roles of SFKs and FAK in CasSD phosphorylation during the early stages (within the first 30 min) of spreading of mouse embryonic fibroblasts on fibronectin. We found that both integrin engagement, which triggered activation of SFK, and anchorage of integrin on the substrate greatly enhanced CasSD phosphorylation. Full-length FAK, independently of its kinase activity, linked Cas to an N-WASP-containing actin polymerization complex. Actin polymerization, but not actomyosin contraction, catalyzed Cas phosphorylation during spreading. Thus, we mapped a pathway leading to Cas phosphorylation through actin polymerization in early cell spreading.

RESULTS**CasSD phosphorylation correlates with cell spreading and adhesion**

Phosphorylation of CasSD is closely linked to the regulation of cell motility. To test the role of Cas in matrix-activated cell spreading, we knocked down Cas using plasmids expressing small hairpin (sh)RNA in mouse embryonic fibroblasts and monitored the phosphorylation of the CasSD after re-plating the cells on fibronectin. Expression of shRNAs against Cas caused a

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substantial reduction in the level of Cas, as well as the level of phosphorylated CasSD, as monitored by immunoblotting using an antibody against total Cas and antibodies specific to Cas that was phosphorylated at residues Y165 and Y410 (pY165 and pY410, respectively) (Fig. 1D). Time-lapse imaging of cells transfected with control shRNA on fibronectin revealed that the majority of the cells exhibited a fast and smooth edge protrusion followed by slower, yet robust, edge activity that is evidenced by strong rearward flow, reminiscent of lamellipodium-driven cell spreading (76% of the cells spread with smooth edge protrusions within 30 min of observation, 16% of the cells spread without forming the robust edge protrusion and 8% of the cells showed no obvious spreading; $n=114$; Fig. 1A,E; supplementary

material Movie 1). The expression of a combination of mouse Cas shRNAs caused a considerable decrease of lamellipodial spreading (22% of the cells with green fluorescent protein (GFP) transfection marker showed lamellipodial-type spreading; $n=100$). In 53% of Cas-shRNA-expressing cells, filopodial extension preceded broader edge protrusion and the amplitude of protrusive activity was substantially reduced. Notably, in 27% of the cells, the edge protrusion between the filopodium-like processes receded quickly, causing rounding up of the cell (Fig. 1B,C,E; supplementary material Movies 2, 3). In the remaining 25% of the cells, protrusive activity seemed to be further reduced and no initial spreading was observed within 30 min, although extension of processes was sometimes seen (Fig. 1E). Thus, consistent with previous reports,

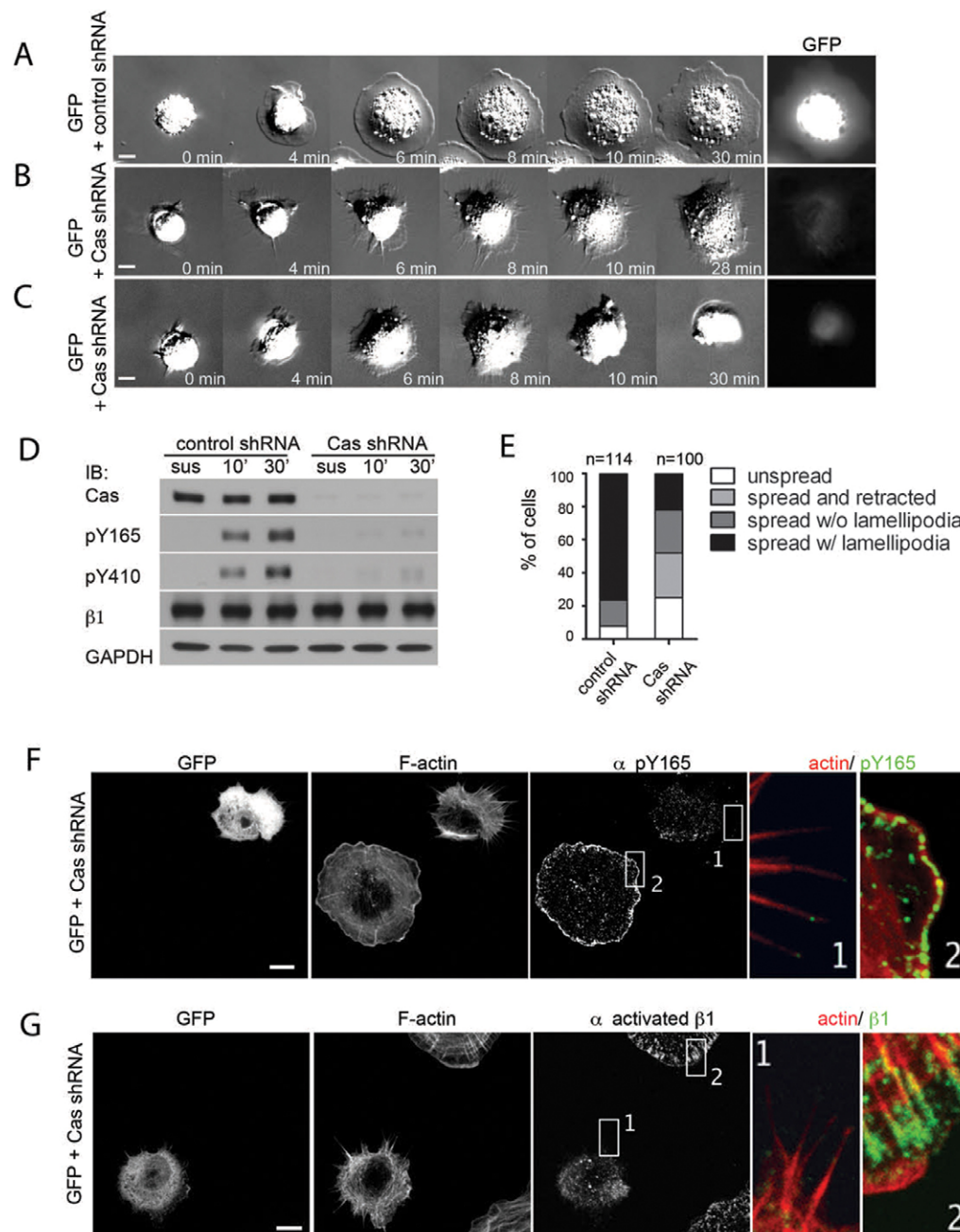


Fig. 1. Inhibition of Cas phosphorylation in fibroblasts causes reduced spreading and loss of lamellipodial protrusions.

(A–C) Time-lapse sequential images showing the spreading on fibronectin of cells that have been transfected with GFP plus control shRNA (A, supplementary material Movie 1) or GFP plus Cas shRNA (B,C; supplementary material Movies 2, 3). Mouse embryonic fibroblasts were transfected with the GFP-expressing plasmid and expression plasmids of nontargeting shRNA or Cas shRNAs. After 72 h of transfection, cells were harvested and plated on fibronectin-coated cover glass for live imaging for 30 min. (D) In the same sample as A–C, reduction of Cas was monitored by western blot (IB) for Cas. Reduction of CasSD phosphorylation was detected using antibodies against the Y165 and Y410 residues. sus, suspension. (E) Summary of the spreading behavior of control-shRNA- and Cas-shRNA-transfected cells in the first 30 min on fibronectin. w/, with; w/o, without. (F) Staining of the actin cytoskeleton showed loss of active edges that correlated with loss of CasSD phosphorylation in early spreading of cells transfected with GFP plus Cas shRNA. Cells were fixed 10 min after plating. The difference in the actin structure can be seen between cells with GFP co-transfection marker (inset 1) or left untransfected (inset 2). Scale bars: 10 μ m. (G) Fibroblasts were fixed as in F and stained with the 9EG7 antibody against activated $\beta 1$ integrin. The Cas-knockdown cell (inset 1) lacked the mature adhesion structure that is observed in untransfected cells (inset 2). Scale bars: 10 μ m.

the depletion of Cas noticeably inhibited smooth lamellipodial protrusions during the early stages of cell spreading.

Immunostaining of early spreading cells confirmed a lack of lamellipodial actin in Cas-shRNA-expressing cells (Fig. 1F). The actin cytoskeleton was reduced to filopodial processes and the arc-shaped stress fibers parallel to the cell edge. Limited staining for pY165 was seen at the tip of the processes (Fig. 1F, inset). Staining cells with a 9EG7 antibody that labels activated $\beta 1$ integrin also failed to detect focal adhesions at the protrusive edge of Cas-knockdown cells, indicating that adhesion formation was defective (Fig. 1G). The spreading morphology was a specific effect of inhibition of Cas signaling as co-transfection of an shRNA-resistant Cas construct tagged with red fluorescent protein (RFP), but not a constitutively inactive RFP-Cas construct in which all tyrosines in the 15 YxxP motifs in CasSD were mutated [RFP-Cas(15F)], rescued the morphology of early spreading cells (supplementary material Fig. S1). Thus, CasSD phosphorylation, active edge protrusion and adhesion are correlated in the spreading of cells on fibronectin.

SFKs downstream of $\beta 3$ integrin phosphorylate CasSD in an anchorage-dependent manner

Both SFKs and Cas are important mediators in adhesion-induced integrin signaling (O'Neill et al., 2000; Shattil, 2005). The C-terminus of Cas harbors binding sites for Src that are important for proper localization and phosphorylation of Cas (Nakamoto et al., 1996; Nakamoto et al., 1997; Fonseca et al., 2004). Consistently, expression of the C-terminal domain of Cas (CasC) negatively influenced CasSD phosphorylation upon cell spreading (supplementary material Fig. S2A). As monitored by phosphorylation of SFK at residue Y416 (pY416), SFK activity increased upon plating of the cells on fibronectin. Inhibition of SFK activity by treatment with inhibitor PP2 enhanced the level of pY416 of SFKs upon spreading (Fig. 2A,D). However, phosphorylation of CasSD at residue Y165 or Y410, upon plating, was severely diminished (Fig. 2A–C). Concomitantly, edge protrusion and lamellipodium formation was also disrupted (Fig. 2E,F). Thus, the kinase activity of SFKs is crucial for early cell spreading and CasSD phosphorylation.

Matrix-induced CasSD phosphorylation not only depends on the kinase activity of SFK but also requires the presence of a substrate of proper rigidity (Kostic and Sheetz, 2006). Previous studies have linked $\beta 3$ integrin to SFK signaling (Felsenfeld et al., 1999; Huveneers et al., 2007). In early spreading cells, $\beta 3$ integrin and activated SFK, recognized by the anti-pY416 antibody, formed clusters that colocalized with each other on the fibronectin-coated surface (supplementary material Fig. S2B). To examine the mechanical role of SFKs in the phosphorylation of CasSD, we compared cells in suspension to adherent cells. Coimmunoprecipitation assays confirmed that a portion of $\beta 3$ integrin interacted with the SFK family member Src independently of adhesion formation, because Src was detected in $\beta 3$ -integrin-containing immunoprecipitated protein complexes in both cells in suspension and adherent cells (Fig. 2G). When the RGD peptide, a soluble ligand for integrin, was added to cells in suspension, there was a substantial increase in SFK activity, as indicated by phosphorylation of residue Y416 (Fig. 2H,I). The rise of SFK activity within 5 min of the addition of RGD was more than 80% of the increase that was induced by fibronectin. A small increase in the basal levels of CasSD phosphorylation was also seen upon addition of RGD to suspension cells which is,

most likely, due to increased SFK activity (Fig. 2H–J). However, the RGD peptide interfered with CasSD phosphorylation in early spreading cells on fibronectin and caused no detectable decrease of SFK activity (Fig. 2H–J). Therefore, although both soluble RGD and immobilized fibronectin readily activate SFKs, phosphorylation of CasSD requires the presence of immobilized fibronectin.

Cas and FAK complex formation is important for CasSD phosphorylation

FAK is a crucial binding partner of Cas and is responsible for phosphorylation of CasSD (Fonseca et al., 2004; Sanders and Basson, 2008). FAK and Cas coimmunoprecipitated with each other in adherent fibroblasts and fibroblasts in suspension (Fig. 3A,B). Coexpressed GFP-Cas and FAK-mCherry colocalized at adhesion sites, where we also detected high levels of CasSD phosphorylation (supplementary material Fig. S3). However, both the adhesion localization pattern of Cas-GFP and the phosphorylation of CasSD were lost when a Cas-binding mutant of FAK (FAK 712-715 P>A; Reiske et al., 1999) was coexpressed. Taken together, these data suggest a requirement for the Cas-FAK interaction for the subsequent Cas phosphorylation (supplementary material Fig. S3). Moreover, knockdown of FAK in the fibroblasts significantly reduced CasSD phosphorylation on fibronectin (Fig. 3C–E), reconfirming the role of FAK in Cas signaling in early fibroblast spreading. Introduction of a dominant-negative form of FAK, a GFP-FRNK construct, into fibroblasts substantially inhibited cell spreading and FAK autophosphorylation at Y397 (Richardson and Parsons, 1996), and decreased phosphorylation of CasSD to a level that was comparable to the FAK-knockdown sample (Fig. 3F–H). Immunostaining confirmed the decrease of phosphorylation of FAK at residue Y397 and Cas at residue Y165 in GFP-FRNK-transfected cells. At the same time, cells transfected with GFP-FRNK lacked a continuous lamellipodium when plated on fibronectin (Fig. 3N,O).

The existing model indicates that autophosphorylation of the FAK Y397 site is important for FAK-mediated CasSD phosphorylation (Ruest et al., 2001). To examine whether the decrease of Cas phosphorylation caused by FRNK was, indeed, due to the lack of the Y397 phosphorylation site on FRNK, we applied the FAK inhibitor PF-228 to block FAK kinase activity and autophosphorylation of Y397 (Slack-Davis et al., 2007). Pre-incubation of cells with PF-228 efficiently inhibited the phosphorylation of FAK on Y397 in cells plated on fibronectin. However, early cell spreading was not affected, nor was phosphorylation of the CasSD on Y165 (Fig. 3I–K). An independent study using another FAK-specific inhibitor also showed that kinase activity of FAK was not required for SFK activity or adhesion-dependent CasSD phosphorylation (Tanjoni et al., 2010). The fact that these results do not support the existing model could reflect the differences between biochemical assays which use overexpressed or endogenous protein levels.

To determine whether FRNK was competing with FAK for binding to Cas, we performed immunoprecipitation of Cas. GFP-FRNK was detected in the immunoprecipitated protein complex. In the same sample, the amount of full-length FAK associated with Cas showed a significant decrease (Fig. 3L,M). Thus, although FAK kinase activity and autophosphorylation of FAK at residue Y397 is not required for Cas signaling, the association of full-length FAK with Cas is important, and FAK might serve as a docking molecule for Cas, leading to phosphorylation of CasSD.

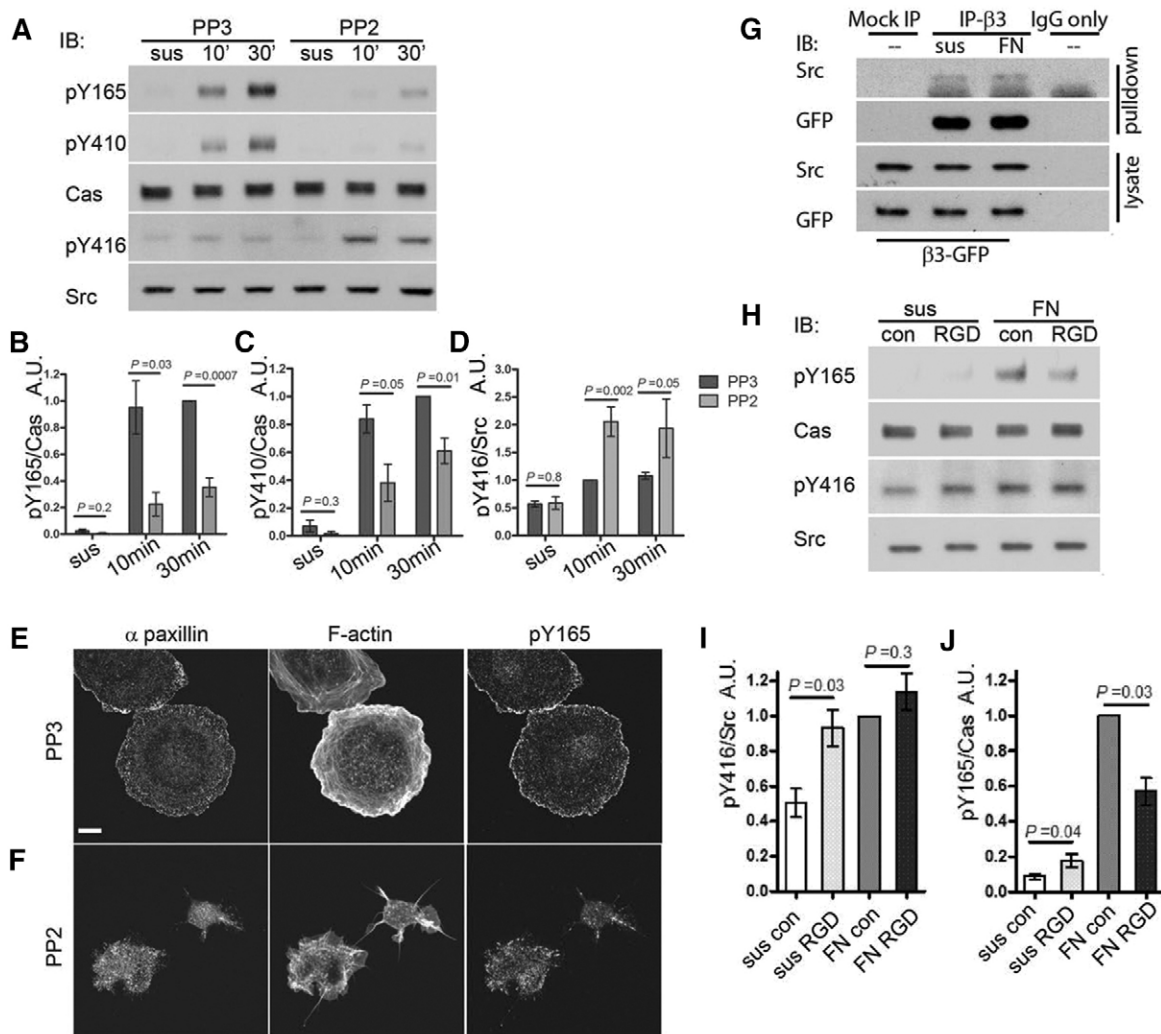


Fig. 2. SFKs downstream of integrins phosphorylate CasSD in an anchorage-dependent manner. (A–D) Inhibition of SFK by use of PP2 diminished fibronectin-induced CasSD phosphorylation at residues Y165 and Y410 but caused hyperphosphorylation of SFK at residue Y416, as shown by western blot (IB) (A). sus, suspension. Cells were pre-incubated in 20 μ M PP2 or its control chemical PP3 for 30 min before being plated on fibronectin-coated dishes (FN). The ratios of (B) pY165 CasSD:total Cas and (C) pY410 CasSD:total Cas in different experiments were normalized against control samples at the 30-min time-point and (D) pY416 Src:total Src ratios against control samples at the 10-min time-point; mean \pm s.d., four repeats. *P*-values were calculated by two-tailed paired Student's *t*-test. A.U., arbitrary units. (E,F) Immunostaining of (E) PP3-treated or (F) PP2-treated early spreading cells. Cells treated with PP2 showed a delay in spreading and defective formation of active protrusions. Sites of limited Cas phosphorylation signal correlated with local actin polymerization (F). Cells were fixed 10 min after spreading initiation. Scale bar: 10 μ m. (G) Interaction of β 3 integrins with Src was detected in cells in suspension and adherent cells. Fibroblasts were transfected with a β 3-integrin–GFP plasmid and harvested 24 h later, kept in suspension for 45 min and then directly lysed with RIPA buffer or plated onto a fibronectin-coated dish for 15 min before lysis. 2 μ l of polyclonal antibody was used to pull down endogenous and overexpressed β 3 integrins. The mock sample was prepared from the lysate of cells in suspension and no antibody was applied. Src associated with the immunoprecipitated (IP) β 3 integrins was detected using an antibody against Src (shown in H). The level of β 3 integrin pulled down was monitored using an antibody against GFP. (H–J) The SFK activity of suspension cells increased in response to RGD soluble peptide. Application of RGD did not affect the SFK activity in early spreading cells although CasSD phosphorylation showed a significant decrease. **P* < 0.05. Fibroblasts kept in suspension were collected by centrifugation and re-suspended in medium containing 1 mM RGD peptide for 5 min before being collected by centrifugation and lysed for western blot (H). The control (con) sample was similarly prepared, except that the RGD peptide was excluded from the re-suspension medium. For adherent samples, fibroblasts were spread on fibronectin for 5 min before the medium was removed and RGD containing medium (or plain medium for control) was applied for another 5 min. Cells were washed once with PBS and lysed directly. Both (I) pY416 Src:total Src and (J) pY165 Cas:total Cas ratios in different experiments were normalized against the adhesion sample treated with control medium; mean \pm s.d., four repeats. *P*-values were calculated by two-tailed paired Student's *t*-test.

Neither actomyosin contraction nor talin-dependent focal adhesions are required for fibronectin-activated CasSD phosphorylation

Because stretching of the cytoskeleton facilitates phosphorylation of CasSD (Sawada et al., 2006), we tested whether myosin contractile activity was required for CasSD phosphorylation. When actomyosin contraction was inhibited by 50 μ M

blebbistatin, neither phosphorylation of residue Y165 nor residue Y410 of CasSD was affected upon cell spreading on fibronectin (Fig. 4A–C). However, treatment with blebbistatin significantly reduced FAK autophosphorylation on residue Y397 and maturation of focal adhesions (Fig. 4A,D,F,H). Immunostaining confirmed the robust phosphorylated Cas signal in blebbistatin-treated cells (Fig. 4E,F).

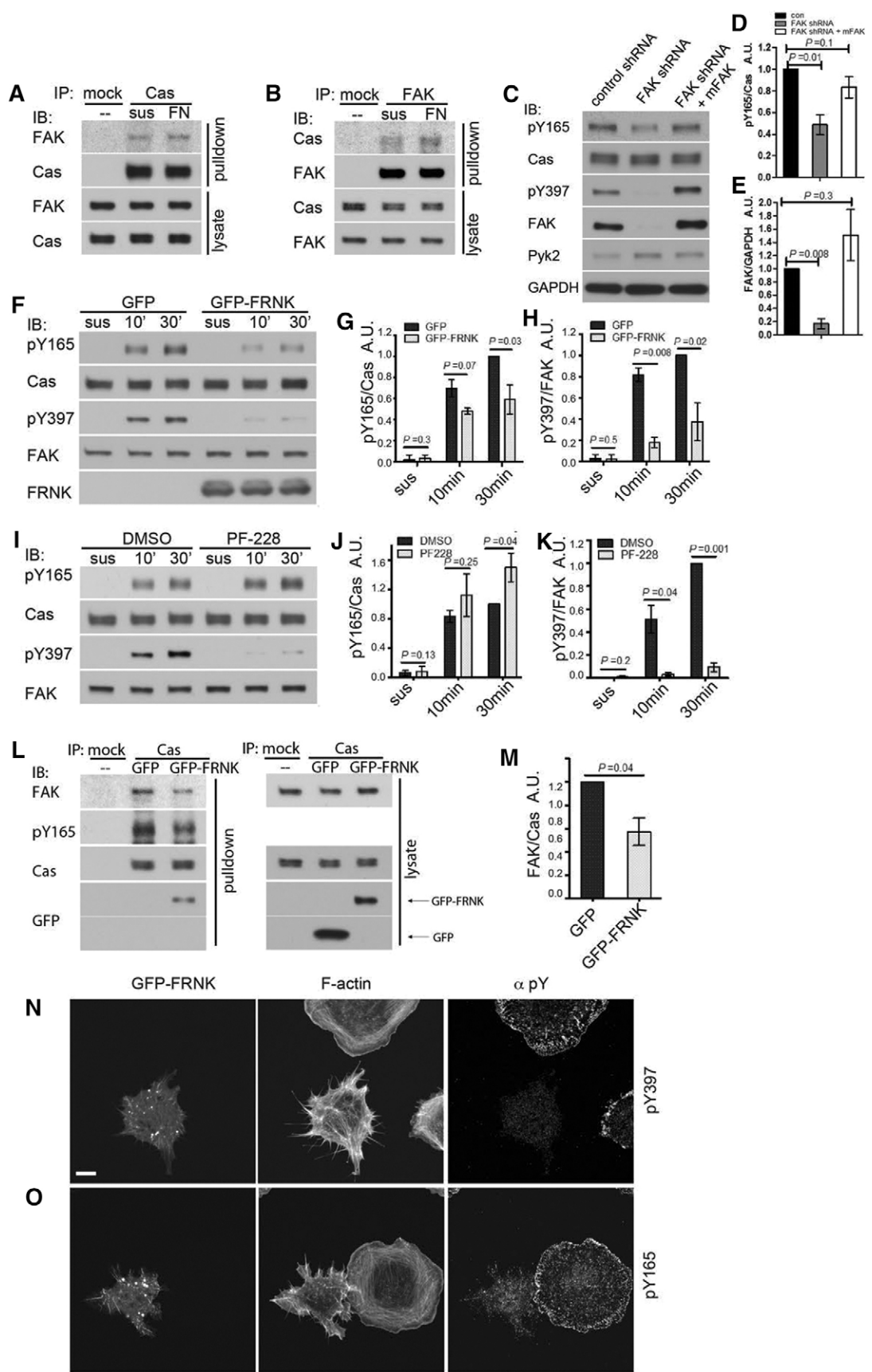


Fig. 3. See next page for legend.

Fig. 3. Formation of FAK-Cas complex is crucial for CasSD phosphorylation. (A,B) FAK and Cas associated with each other in suspension and adherent cells. Fibroblasts were harvested, kept in suspension (sus) for 45 min and directly lysed with RIPA buffer or plated on fibronectin-coated dishes (FN) for 15 min before lysis. 5 μ g of combined polyclonal and monoclonal antibodies against Cas (A) or against FAK (B) were used to pull down Cas and FAK from the lysates. The mock sample was prepared from the lysate of cells in suspension (sus) and lacked antibody. FAK and Cas associated with the immunoprecipitated (IP) complex were detected with monoclonal antibodies against FAK or Cas in the western blot assays (IB). Loading of pulldown samples was adjusted so that lysate content of Cas and FAK levels were comparable between adherent cells and cells in suspension. (C–E) Knockdown of FAK affected CasSD phosphorylation in fibroblasts. Plasmids encoding control shRNA, FAK shRNA, or FAK shRNA and an shRNA-resistant mouse FAK (mFAK) were transfected into fibroblasts by electroporation. After 72 h of transfection, cells were harvested and left in suspension for 45 min before being plated onto fibronectin-coated dishes for 10 min. Cells were directly lysed for western blot assay. (D) The ratios of pY165 Cas to total Cas, and (E) FAK to GAPDH were normalized against the control shRNA sample. Significant decrease of CasSD pY165 signal was seen with efficient FAK depletion, $*P < 0.05$. A.U., arbitrary units. (F–H) Expression of FRNK interfered with CasSD phosphorylation. Fibroblasts were transfected with a GFP or GFP–FRNK construct. 16 h after transfection, cells were harvested and kept in suspension for 45 min. Cells were then plated on fibronectin and subjected to direct western blot (F). (G) pY165 Cas:total Cas ratios in different experiments were normalized against a GFP sample at the 30-min time-point. (H) pY397 FAK:total FAK ratios in different experiments were normalized against a GFP sample at the 30-min time-point. (I–K) Inhibition of FAK kinase activity did not affect CasSD phosphorylation. Cells were pre-incubated in DMSO or 5 μ M of FAK inhibitor PF-228 for 30 min before being plated on fibronectin and being subjected to western blot assay (I). (J) pY165 Cas to total Cas ratios in different experiments were normalized against a DMSO sample at the 30-min time-point. (K) pY397 FAK:total FAK ratios were normalized against the DMSO-treated sample at the 30-min time-point. (L,M) FRNK associated with Cas and disrupted the FAK–Cas interaction. Cells transfected with GFP or GFP–FRNK were plated on fibronectin for 15 min before being lysed in RIPA buffer. Cas was immunoprecipitated as in A, and associated FAK was detected using a monoclonal antibody against FAK, and FRNK was detected by using an antibody against GFP in western blot assays (L). The mock sample was prepared from the untransfected cells and no antibody was applied for immunoprecipitation. FAK:Cas ratios in different pulldown experiments were normalized against the values of GFP samples (M). Significant decrease of full-length FAK:Cas ratio was seen, $*P < 0.05$. (N,O) Immunofluorescence showed that phosphorylation of FAK at residue Y397 and Cas at residue Y165 was disrupted in GFP–FRNK-transfected cells. Cells were transfected as in F and fixed 12 min after plating. Untransfected cells in the same pool served as control for staining. Scale bar: 10 μ m. Mean \pm s.d. are shown from three repeats. P -values were calculated by two-tailed paired Student's t -test.

Talin is important for the formation of focal adhesions and coupling of contraction to the matrix (Zhang et al., 2008). We depleted talin2 in talin1^{−/−} fibroblasts to test whether CasSD phosphorylation was dependent on talin. Plating talin-depleted cells on fibronectin induced an initial increase of pY165 and pY410 comparable to the level of increase in the control cells at the 10-minute time-point (Fig. 4I–K). At later time-points, pY165 and pY410 levels decreased in the absence of talin, probably because talin-depleted cells could not form stable adhesions and rounded-up after initial spreading (Zhang et al., 2008). Fixation and immunostaining of early spreading talin-depleted cells revealed no correlation between total talin levels and the intensity of pY165 staining. No talin-based focal adhesions were observed in talin-depleted cells; however, we found Cas Y165 phosphorylation at the distal tip of actin bundles at the leading edge (Fig. 4L). A similar localization pattern was also

observed for active SFK (pY416) but not pY397 FAK or paxillin (supplementary material Fig. S4). Thus, CasSD phosphorylation appears to be an earlier step in integrin signaling than talin-dependent adhesion formation.

CasSD phosphorylation is induced by actin polymerization activity and Cas is associated with a FAK–N-WASP complex

The localization of pY165 Cas underneath the lamellipodium and at the tip of actin bundles indicated that there was an association of phosphorylated Cas with actin structures (Fig. 4E,F,L). To understand the role of actin dynamics in CasSD phosphorylation, we applied low doses of cytochalasin D (200 nM) or latrunculin A (150 nM) to cells in suspension and followed the CasSD phosphorylation profile after re-plating the cells on fibronectin. At these dosages, both drugs inhibit the growth of F-actin at the cell edge (de Oliveira and Mantovani, 1988; Urbanik and Ware, 1989) and remarkably slowed the protrusion of the cell edge in our assay. At 12 and 30 min after plating, decreased phosphorylation of residues Y165 and Y410 was observed in drug-treated cells, when compared with controls (Fig. 5A–C). Staining for F-actin confirmed the disruption of the actin cytoskeleton by the inhibitors (Fig. 5E–G). When treated with cytochalasin D, cells lacked a clearly developed lamellipodium. Nevertheless, cells were able to form actin-based processes and the remaining pY165 CasSD signal lined these structures (Fig. 5F, inset). When latrunculin A was applied, sporadic actin polymerization was observed at the cell edge but very little pY165 signal was seen (Fig. 5G).

To identify the molecules that coupled Cas to polymerizing actin, we examined FAK and its binding partners, which might form a potential link to the actin cytoskeleton. The FERM domain of FAK interacts with N-WASP, an activator of actin polymerization and a regulator of a full range of actin-based processes (Takenawa and Suetsugu, 2007; Wu et al., 2004). By coimmunoprecipitation assay, we confirmed that there was an interaction between N-WASP and FAK that was independent of adhesion (Fig. 5H). In the same experiment, we also detected Cas in the N-WASP immunoprecipitated protein complex in samples taken from adherent cells and cells in suspension. N-WASP co-precipitated in a Cas pulldown assay, which further confirmed the existence of a Cas–N-WASP complex (Fig. 5I). We also introduced FRNK into the cell and discovered that association of Cas with N-WASP was significantly decreased, as measured by the amount of Cas that coimmunoprecipitated with N-WASP; however, the N-WASP–FAK association was not affected (Fig. 5J–L). Binding of N-WASP to FAK has been mapped to the first 400 amino acids of FAK (Wu et al., 2004), a region that is absent in FRNK. Our observation indicates that the presence of full length FAK, which interacts with both Cas and N-WASP, is required for the formation of a Cas–FAK–N-WASP complex, and disruption of this complex by FRNK overexpression correlates with a decrease in CasSD phosphorylation.

In spreading fibroblasts, GFP–N-WASP localized to the distal edge of the lamellipodium, similar to the staining for N-WASP (Fig. 5M; supplementary material Fig. S5). In cells treated with cytochalasin D and latrunculin A, where cell-edge protrusions were substantially affected, N-WASP was observed at the remaining actin-rich structures (Fig. 5N,O; supplementary material Fig. S5). At actively protruding edges of cells, N-WASP colocalized with phosphorylated Cas at the matrix-cell interface, consistent with its involvement in Cas phosphorylation in early spreading cells (Fig. 5M–O, inset).

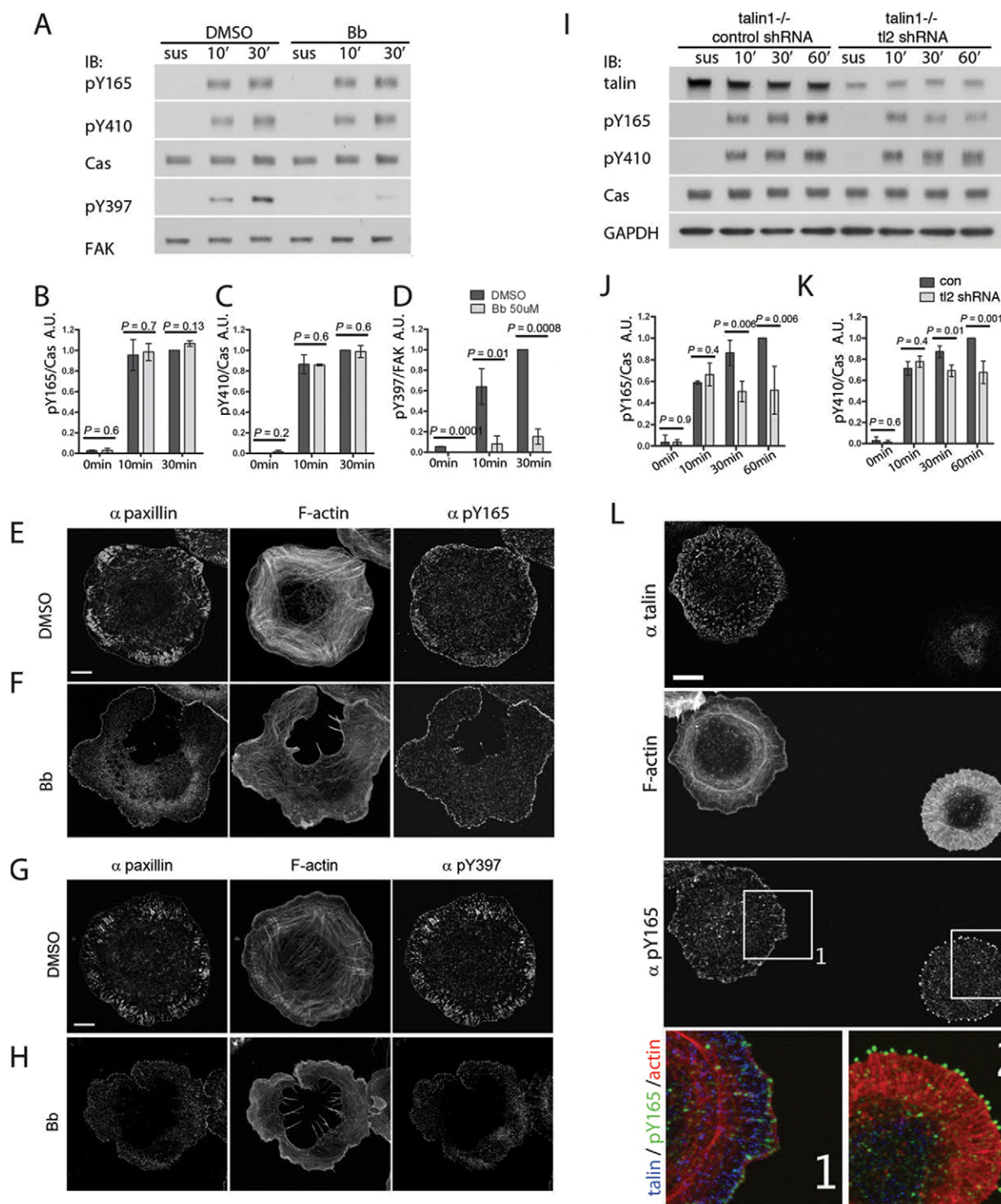


Fig. 4. Neither actomyosin contractility nor talin-dependent focal adhesion is required for CasSD phosphorylation. (A–D) CasSD phosphorylation during spreading on fibronectin did not decrease with the treatment of blebbistatin (Bb). However, FAK phosphorylation at residue Y397 was significantly impaired ($*P<0.05$). Cells were pre-incubated in medium containing DMSO or 50 μM blebbistatin for 30 min and plated on fibronectin-coated dishes for the indicated time before being lysed for western blot assay (IB). sus, suspension. A.U., arbitrary units. (E–H) F-actin staining confirmed that stress fibers were missing in the cells treated with blebbistatin (F,H). The pY165 signal of Cas was comparable in control and treated cells (E,F). FAK phosphorylation at residue Y397 was greatly reduced with loss of focal adhesion structure in blebbistatin-treated cells (G,H). All samples were fixed 20 min after plating. (I–K) Western blots showed comparable CasSD phosphorylation at residue Y165 in talin-deficient cells 10 min after plating on fibronectin although levels of pY165 decreased at later time-points (I). The profile of phosphorylation at residue Y410 was similar to that for residue Y165, except that a smeared signal at a lower molecular mass was observed at later time-points. (J) pY165 Cas:total Cas and (K) pY410 Cas:total Cas ratios in different experiments were normalized against control samples (con) at the 60-min time-point. Depletion of talin2 in talin1^{-/-} fibroblasts was examined by total talin antibody 8d4. tl2, talin2. Means \pm s.d., three repeats. Two-tailed paired Student's *t*-test was performed. (L) Immunostaining with talin antibody and an antibody against Cas phosphorylated at residue Y165 confirmed that reduction of total talin did not affect CasSD phosphorylation when the cell was still spread. Cells were fixed 15 min after plating. Scale bars: 10 μm.

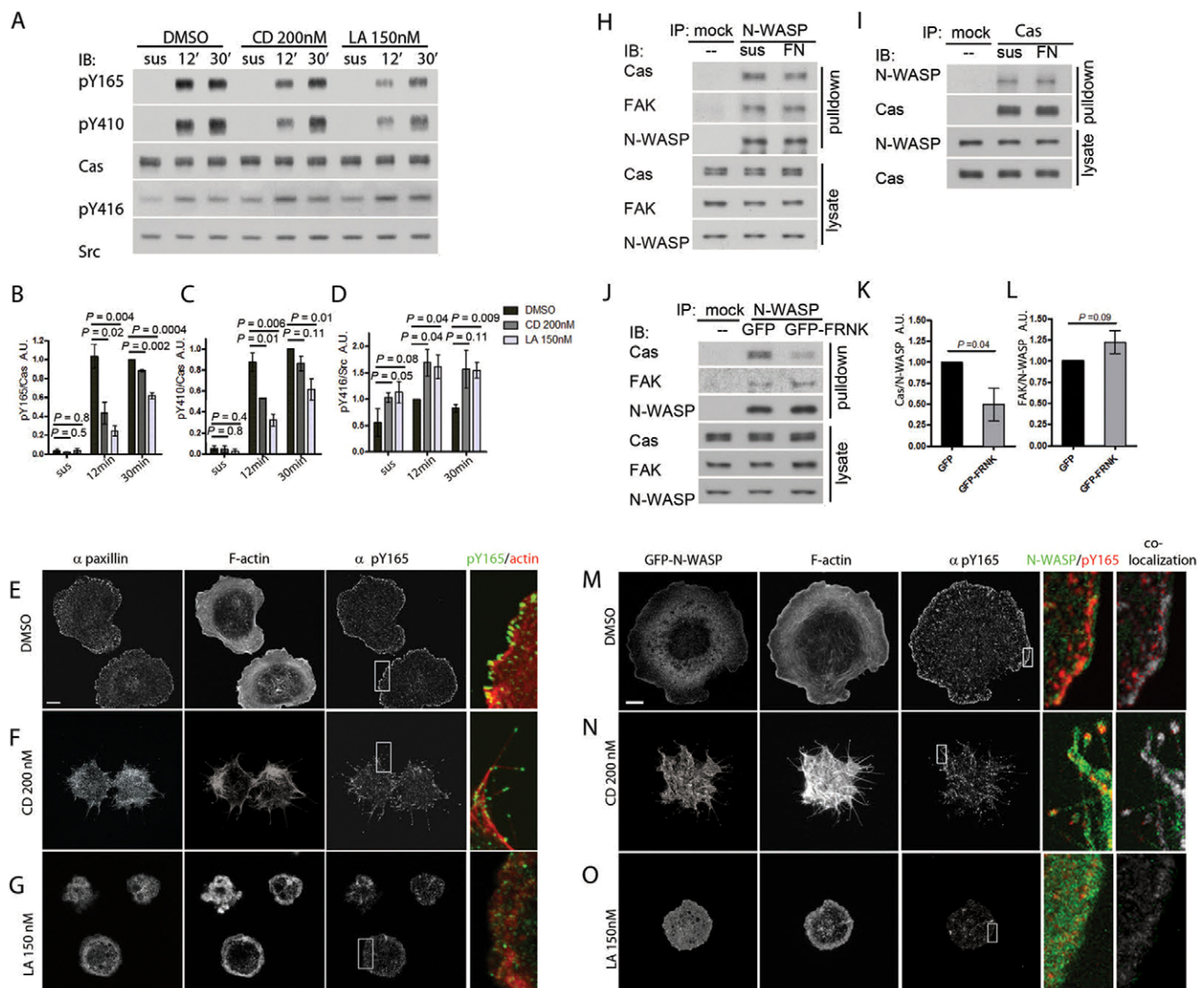


Fig. 5. CasSD phosphorylation is dependent on actin polymerization activity and Cas is associated with the FAK–N-WASP complex. (A–D) Fibroblasts in suspension (sus) were pre-incubated in medium containing DMSO, 200 nM cytochalasin D (CD) or 150 nM latrunculin A (LA) for 30 min before being plated on fibronectin (FN). At the indicated time, cells were lysed with sample buffer for western blot assay (IB) (A). CasSD phosphorylation and SFK activity were monitored using antibodies against pY165 Cas, pY410 Cas and pY416 Src. pY165 Cas:total Cas and pY410 Cas:total Cas ratios of each experiment were normalized against the DMSO sample at the 30-min time-point. The pY416 Src:total Src ratios were normalized against the DMSO sample at the 10-min time-point. A.U., arbitrary units. (E–G) Fixation and immunostaining of spreading cells (12 min after plating) confirmed that the CasSD phosphorylation decreased during cell spreading, when actin polymerization was inhibited. The treated cells spread to their normal area at the 30-min time-point; however, the cytoskeleton structure was altered (not shown). The remaining phosphorylated Cas signal in cytochalasin-D-treated cells, as well as certain patterns observed in control cells, were correlated with sites of actin process formation (E,F, inset). The areas indicated by the white boxes are shown in the inset images. (H,I) N-WASP was associated with Cas and FAK in suspension and adherent cells. Suspension and adherent cell lysates were prepared as in Fig. 3A,B. 2 μ g of polyclonal antibody against N-WASP (H) and 5 μ g of combined antibodies against Cas (I) were used in the immunoprecipitation (IP). FAK and Cas were detected using monoclonal antibodies and N-WASP was detected using the same antibody that had been used for immunoprecipitation. The mock sample was prepared as in Fig. 3A,B. (J–L) Expression of FRNK disrupted Cas–N-WASP association but not FAK–N-WASP interaction. Cell lysates were prepared as in Fig. 3L and the antibody against N-WASP was applied in the pull-down assay. The amount of Cas pulled down with N-WASP IP showed a clear decrease when FRNK was expressed, $*P<0.05$. Mean \pm s.d., three repeats. Two-tailed paired Student's *t*-test was performed. (M–O) N-WASP localized at the distal edge of actin protrusions. Cells were transfected with a GFP–N-WASP plasmid for 24 h and treated and plated as in E–G. The areas indicated by the white boxes are shown in the inset images. Colocalization (inset, shown in white) was detected by ImageJ colocalization assays. Scale bars: 10 μ m.

To test whether N-WASP played a direct role in CasSD phosphorylation, we inhibited N-WASP with wiskostatin, a small molecule that stabilizes N-WASP in an autoinhibited conformation (Peterson et al., 2004). Pre-incubation of cells in 10 μ M wiskostatin severely interfered with robust cell spreading and reduced CasSD phosphorylation on fibronectin, as detected by the antibody against pY165 in western blots (Fig. 6A,B).

Knockdown of N-WASP consistently decreased CasSD phosphorylation (Fig. 6C–E), although, presumably due to residual N-WASP and alternative pathways that activate Cas, the phenotype was somewhat milder. Imaging of immunofluorescence revealed that the cells with knockdown of N-WASP were delayed in forming robust lamellipodia at the first 10 min of spreading, compared with control cells (Fig. 6F).

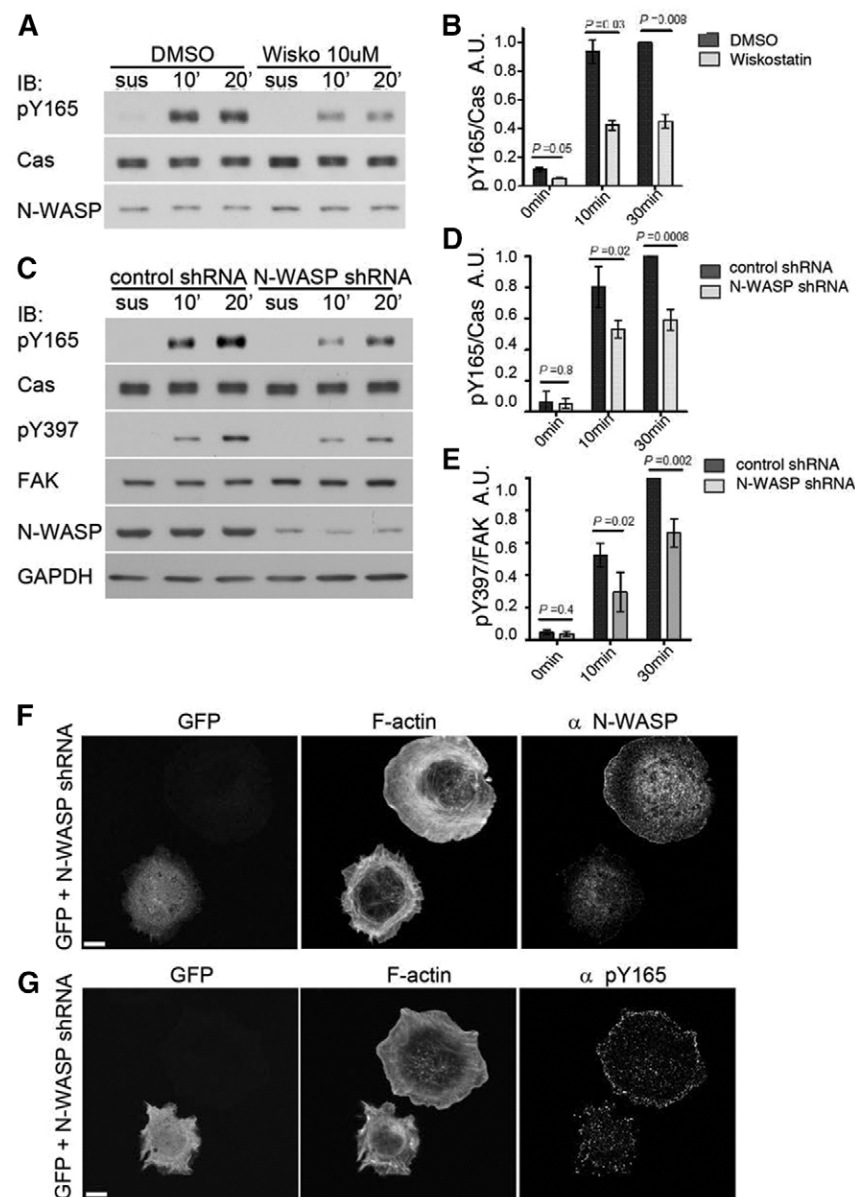


Fig. 6. N-WASP is involved in the mechanism leading to CasSD phosphorylation. (A,B) Inhibition of N-WASP by wiskostatin disrupted CasSD phosphorylation. Cells were pre-incubated in DMSO or 10 μ M wiskostatin for 20 min before being plated on fibronectin and lysed for western blot (IB). The ratio of pY165 Cas:total Cas of control cells after 20 min of spreading was set as 1 and the ratio of other time-points were normalized accordingly. Mean \pm s.d., two repeats, two-tailed paired Student's *t*-test. A.U., arbitrary units; sus, suspension. (C–E) Knockdown of N-WASP decreased CasSD phosphorylation upon cell spreading on fibronectin. FAK phosphorylation at the Y397 site also decreased. Cells expressing scrambled or N-WASP-specific shRNA were harvested 2 days after transfection, re-suspended in serum-free medium and plated on fibronectin for the indicated time before being lysed for western blot assay (C). The ratios of pY165 Cas:total Cas and pY397 FAK:total FAK were normalized as in B. Mean \pm s.d., four repeats, two-tailed paired Student's *t*-test. (F,G) Cells co-transfected with GFP and N-WASP shRNA showed delayed spreading at the early time-point. Cells were plated on fibronectin for 10 min before being fixed and stained for N-WASP and pY165 Cas. Scale bars: 10 μ m.

Accordingly, robust pY165 staining at the leading edge was decreased (Fig. 6G). To further confirm the role of N-WASP in Cas signaling, we also inhibited the Arp2/3 complex, the downstream effector of N-WASP, with the small-molecule inhibitor CK-666. Treatment with CK-666 affected early cell spreading and phosphorylation of the CasSD was decreased (supplementary material Fig. S6). Thus, N-WASP activity is, indeed, involved in the pathways leading to CasSD phosphorylation in early cell spreading.

DISCUSSION

The role of Cas in cell spreading and motility has been suggested in several studies (Honda et al., 1999; Sanders and Basson, 2005). Here, we show that Cas is crucial for the activation of sustained cell edge protrusion. Edge protrusion in early cell spreading is driven by lamellipodial actin polymerization that correlates with Rac1 activation and is affected by the inhibition of CasSD phosphorylation (Sharma and Mayer, 2008). Furthermore, Rap1, which promotes adhesion formation, has also been reported to be

activated downstream of CasSD phosphorylation (Boettner and Van Aelst, 2009; Sawada et al., 2006). Changes in small GTPase activities might explain the defective cell spreading observed upon knockdown of Cas.

Stretching of the cytoskeleton has been shown to increase Cas phosphorylation (Tamada et al., 2004; Sawada et al., 2006), which led to the speculation that cytoskeletal contraction might drive the phosphorylation of Cas under physiological conditions. However, neither actomyosin contraction nor talin-dependent focal adhesion formation seems to be involved in substrate-induced CasSD phosphorylation in early cell spreading. SFKs bind to integrins and are the major kinases for CasSD phosphorylation in fibroblasts (Arias-Salgado et al., 2003; Sakai et al., 1997). Disruption of integrin binding to immobilized fibronectin, by use of a soluble RGD peptide, reduces CasSD phosphorylation in spreading cells without affecting the SFK kinase activity. In addition, linking Cas to the N-WASP-containing actin polymerization complex seems indispensable for proper CasSD phosphorylation. Phosphorylation of CasSD decreases after the inhibition of N-WASP and the

downstream Arp2/3 complex, general actin polymerization activity, or disruption of the Cas–FAK–N-WASP complex. Thus, phosphorylation of CasSD not only requires SFK kinase activity but also depends on the mechanical coupling of Cas to dynamic actin filament assembly.

In addition to the present findings, others have reported that both FAK and SFKs are crucial for CasSD phosphorylation (Fonseca et al., 2004; Hamamura et al., 2008; Sanders and Basson, 2008). Results from an earlier study of FAK mutants indicate that the ability of overexpressed FAK to enhance CasSD phosphorylation relies on the autophosphorylation of FAK residue Y397 (Ruest et al., 2001). However, in early spreading fibroblasts, phosphorylation of FAK residue Y397 did not correlate with the CasSD phosphorylation profile (Fig. 4A) and further inhibition of this site with a pharmacological inhibitor of FAK did not affect CasSD phosphorylation. Similarly, another study of pharmacological inhibition of FAK also reveals that CasSD phosphorylation is independent of phosphorylation of FAK residue Y397 (Tanjoni et al., 2010). Compared with the previous approach, which relied heavily on overexpression systems, our approach allowed us to acutely manipulate the kinase activity without disrupting the endogenous protein level. Disruption of CasSD phosphorylation in early cell spreading upon expression of FRNK suggests that an alternative model is needed to explain the role of FAK in Cas phosphorylation – namely, that binding of FAK to Cas provides a physical link, leading to Cas phosphorylation.

FAK binds to several actin-polymerization-related proteins that might couple Cas to the leading edge, including N-WASP (Serrels et al., 2007; Wu et al., 2004). In our study, N-WASP was present in the same complex as Cas, whether or not adhesions were present. In cancer cells, N-WASP plays an indispensable role in the assembly of invadopodia, in which phosphorylated Cas is also detected (Albiges-Rizo et al., 2009; Alexander et al., 2008). N-WASP has been shown to interact with integrins and requires phosphorylation by SFKs for its full activity, making N-WASP a possible early responder to integrin signaling (Sturge et al., 2002; Suetsugu et al., 2002). We show here that inhibition or depletion of N-WASP interferes with CasSD phosphorylation in the early spreading of fibroblasts. Although other linkers, that bridge the Cas–FAK complex to the actin cytoskeleton might also be involved in CasSD phosphorylation (Poullet et al., 2001), the association of Cas and N-WASP appears important for Cas phosphorylation in early cell spreading.

Our previous work has demonstrated that initial fast cell-spreading takes place before an increase in actomyosin contractility and focal adhesion formation (Giannone et al., 2004; Zhang et al., 2008). Talin is needed for formation of focal adhesions by cells undergoing actomyosin contraction on the substrate; however, talin is not required for CasSD phosphorylation at early stages. Here, we show that depletion of Cas substantially affects the amplitude of early fast cell-spreading and diminishes adhesion, a phenotype that is stronger than that observed upon depletion of talin. Cas-activated Rap1 and its downstream effector RIAM are necessary for the release of autoinhibition of talin, to promote the binding of talin to integrin $\alpha_{IIb}\beta_3$ and the subsequent formation of adhesions (Han et al., 2006). Thus, activation of Cas might be an important step that is upstream of talin in integrin signaling. Recently, FAK has been shown to be involved in the adhesion-regulated inside-out integrin activation (Michael et al., 2009). Given that FAK plays a crucial role in Cas signaling, it remains to be tested whether Cas

and FAK are involved in the same signaling pathway as that controlling integrin activity.

Our results predict that surface-bound fibronectin catalyzes the activation of integrin–SFK–Cas signaling in fibroblasts during early spreading. Although other components of the cytoskeleton that bind to Cas are likely to modulate Cas signaling (Geiger, 2006), our results indicate that, in early cell spreading, FAK and SFKs are the major binding partners of Cas leading to CasSD phosphorylation. Previous studies link the Cas-interacting proteins and Cas phosphorylation to the sensing of substrate rigidity (Alexander et al., 2008; Jiang et al., 2006; Kostic and Sheetz, 2006). In our knockdown experiments, we detected a decrease of Cas phosphorylation only after a strong reduction of Cas levels, suggesting that only a small fraction of Cas is phosphorylated in early spreading cells. Cas is also highly dynamic, which made it resistant to multiple single-molecule imaging approaches. It has been reported recently that, in early spreading of fibroblasts on fluid lipid bilayers, integrin clustering causes actin-based outwards movement (Yu et al., 2011). Thus, there is a pushing force originating from the actin polymerization activity at early integrin clusters (Roca-Cusachs et al., 2012) that might provide an explanation for the involvement of Cas in the translation of mechanical properties of the environment into biochemical signals (Sawada et al., 2006). A working model, summarizing our data and previous findings, suggests that, in early spreading cells, the FAK–N-WASP complex couples the N-terminal SH3 domain of Cas to polymerizing actin at the protruding edge. Active SFKs in the nascent integrin clusters then bind to Cas at the C-terminus of the CasSD and exert a restraining force on Cas, causing exposure and phosphorylation of the CasSD domain. These events prime the sites of local Cas signaling for sustained spreading and the formation of adhesions.

MATERIALS AND METHODS

Cell culture

Immortalized mouse embryonic fibroblasts (von Wichert et al., 2003) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS). The talin1^{−/−} fibroblastic cell line (Priddle et al., 1998) was maintained in DMEM/F-12 medium (Gibco) supplemented with 15% FBS.

Constructs and transfection

Constructs expressing shRNA against mouse p130Cas (NM_001198839) with the inserted target sequences 5'-CGTGAGGAAACCTATGATGTA-3' and 5'-GCGTGAGGAAACCTATGATGT-3' in the pLKO.1-puro vector were purchased from Sigma and used in combination in equal amounts. A matching vector with a nontargeting sequence inserted was also purchased from Sigma and used as negative control for the Cas-knockdown experiments. The talin2-shRNA-expressing construct and the negative control shRNA construct have been described previously (Zhang et al., 2008). Mouse N-WASP (NM_028459), specific sequence 5'-GACGAGATGCTCCAAATGG-3', and scrambled negative control sequence 5'-GACCAGATGCACCATATCG-3' were cloned into Ambion pSILENCER H1 shRNA expressing vector according to the manufacturer's instructions. To make shRNA against mouse FAK (NM_007982), the FAK-specific sequence 5'-CAATGGAACGAGTA-TTAAA-3' was cloned into pSILENCER H1 vector as described above and a negative control plasmid encoding an shRNA, whose sequence is not found in the mouse databases, was provided by Ambion. To make shRNA-resistant mouse FAK (mFAK), three silent mutations corresponding to amino acids R37, V38 and L39 in mouse FAK were introduced by mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene). Primer 5'-GTCCCCTGGTGCAATGGAA-CGCGTCTTGAAGGTCTTCATCATTTTGAAA-3' and complementary reverse primer were designed on the manufacturer's website. FAK P>A

was created by site-directed mutagenesis of proline 712 and proline 715 to alanine with the forward primer 5'-GGATCAGATGAAGCTGCTCCCA-AGGCCAGCAGGC-3' and the complementary reverse primer. RFP-Cas and RFP-Cas(15F) have been described previously (Sawada et al., 2006). To make Cas-shRNA-resistant RFP-Cas and RFP-Cas(15F) constructs, three silent mutations were introduced into the original RFP-Cas and RFP-Cas(15F) constructs at sites corresponding to E328, E329, T330 using the QuikChange mutagenesis kit. The forward primers used were 5'-GGCCC-CTGCTGCGTGAAGAGACATATGATGTACCTCCTGC-3' for RFP-Cas and 5'-GGCCCCTGCTGCGTGAAGAGACATTTGATGTACCTCCTGC-3' for RFP-Cas(15F), along with the complementary reverse primers. FRNK and GFP-FRNK constructs were a gift from J. Thomas Parsons (University of Virginia, Charlottesville, VA). GFP-N-WASP was as characterized previously (Merrifield et al., 2004). A GFP-Cas C-terminus-domain plasmid (GFP-CasC) was constructed by ligating the rat Cas sequence, corresponding to amino acids 515–969, to the GFP expression vector pEGFP-C3. All plasmids were authenticated by sequencing. All DNA constructs were transfected into wild-type or talin1^{-/-} fibroblasts using the Amaxa nucleofactor system. 5–10 µg of DNA was used per reaction (1E6 cells).

Cover-glass coating and live-imaging microscopy

Silanized cover glasses or culture dishes were coated with human plasma fibronectin (10 µg/ml) (Roche) overnight at 4°C. For live imaging of cell spreading, cells transfected with the indicated construct were trypsinized and resuspended in culture medium for 45 min at 37°C to allow for recovery. Cells were then sealed in a live-imaging chamber, with the bottom cover glass coated with fibronectin, and mounted onto a motorized 37°C stage. Differential interference contrast images were taken on an Olympus IX81 fluorescence microscope with a ×20, numerical aperture 0.7, objective.

Chemicals, antibodies and immunofluorescence staining

Blebbistatin, wiskostatin, PP3 and PP2 were obtained from Calbiochem. GRGDTP peptide was from Sigma. CK-666 was from Millipore. FAK inhibitor PF-228 was purchased from Tocris Bioscience. Antibodies against the following proteins were used: talin (talin1 and talin2, monoclonal clone 8d4, Sigma), GAPDH (Santa Cruz Biotechnology), p130Cas (raised in goat, Santa Cruz), p130Cas (raised in rabbit, polyclonal, GeneTex), p130Cas (monoclonal, BD Transduction), paxillin (BD Transduction), FAK (which does not recognize FRNK, BD Transduction), Src (mAb327, Calbiochem), phospho-FAK (pY397, Biosource International), FAK (polyclonal recognizing full-length FAK and FRNK, Invitrogen), phospho-FAK (pY861, Invitrogen), phospho-Src (pY416, Cell Signaling), phospho-p130Cas (PY165, Cell Signaling), phospho-p130Cas (pY410, Cell Signaling), integrin β3 (polyclonal, Chemicon), active β1 integrin (clone 9EG7, BD Pharmingen), and N-WASP (affinity-purified, polyclonal, raised in rabbit, a gift provided by Marc W. Kirschner at Harvard Medical School). Rhodamine-phalloidin and secondary antibodies were from Molecular Probes.

For immunofluorescence, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Labeling with primary antibody was performed at 4°C overnight and labeling with secondary antibody was performed at room temperature for 45 min. Phalloidin labeling was performed concomitantly with secondary antibody labeling. Images were taken on an Olympus Fluoview FV500 laser scanning confocal microscope with argon 488 nm, HeNe-G 543 nm and HeNe-R 633 nm beams. Images of immunofluorescence were reconstructed from confocal z-slices.

Western blot, immunoprecipitation and quantification

For direct western blots, transfected cells were trypsinized and incubated in suspension for 45 min before being plated on fibronectin-coated culture dishes. At the indicated times, cells were washed once with PBS and lysed in 1× sample buffer [2% SDS, 80 mM Tris-HCl pH 6.8, 10% glycerol and 5% β-mercaptoethanol]. All cell lysates were heated before being loaded onto 4–20% gradient polyacrylamide gels (Lonza). The protein was then transferred to Optitran reinforced nitrocellulose membrane (Whatman). The membrane was blocked with 5% dry milk in TBS with Tween-20 and

incubated with primary antibody overnight at 4°C. The membrane was then incubated for 1 h at room temperature with anti-mouse- or anti-rabbit-HRP-conjugated IgG (Jackson ImmunoResearch). A signal was detected with enhanced chemiluminescence western blotting detection reagents (Amersham Biosciences) on Denville autoradiography film. Signal quantification was performed with ImageJ. For immunoprecipitation, 3E5 cells in suspension or adhered to fibronectin-coated dishes were washed twice with ice-cold PBS and lysed in RIPA buffer [1% NP-40, 150 mM NaCl, 10 mM NaP pH 7.2, 500 µl 5 mM NaF, 5 mM Na₃VO₄ 5 mM and 1 complete mini protease inhibitor tablet (Roche) per 10 ml]. Debris was discarded after the lysates were centrifuged for 20 min at 13793 g. The cleared lysates were either incubated with the indicated antibody (5 µg of antibody against Cas or FAK, 2 µg of NWASP polyclonal antibody or 2 µl of antibody against β3) or without antibody (mock) at 4°C for 3 h. Protein A/G agarose beads (Santa Cruz Biotechnology) were then added to each of the samples for incubation overnight. Beads were later collected by centrifuging at 735 g and washed three times with ice-cold RIPA buffer. Beads were then heated in 2× sample buffer and adjusted to 1× before loading onto 4–20% gradient polyacrylamide gel for assay by western blot.

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Competing interests

The authors declare no competing interests.

Author contributions

X.Z., S.W.M., T.I., and M.P.S. designed research; X.Z., S.W.M. and T.I. performed experiments; X.Z. analyzed data; X.Z. and M.P.S. wrote the paper.

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Supplementary material

Supplementary material available online at <http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.134692/-DC1>

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